Eur Respir J 2006; 27: 1152–1159
DOI: 10.1183/09031936.06.00142905

Early rise in exhaled nitric oxide and mast cell activation in repeated low-dose allergen challenge

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ABSTRACT: Repeated low-dose allergen inhalation challenge mimics natural allergen exposure, providing a model for early mechanisms in the triggering of asthma. The current authors performed a controlled study to evaluate the time course of changes in exhaled nitric oxide fraction ($F_{e,NO}$) and urinary biomarkers of airway inflammation.

Eight subjects with mild allergic asthma completed two 7-day repeated low-dose challenge periods, with diluent and allergen, respectively. Subjects were symptom free at inclusion and were investigated when not exposed to specific allergen. Pulmonary function and symptoms were followed, and $F_{e,NO}$ and urinary mediators were correlated to changes in airway responsiveness to histamine and adenosine.

Despite no change in pulmonary function (forced expiratory volume in one second mean±SEM fall 0.3±0.7 *versus* 0.6±1.0%, for diluent and allergen, respectively) and no asthma symptoms, repeated allergen exposure, in contrast to diluent, caused significant increases in histamine responsiveness (2.3 doubling doses), an early and gradual increase in $F_{e,NO}$ (up to a doubling from baseline) and a small increase in the mast cell marker 9α11β-prostaglandin F_2 after adenosine challenge.

In conclusion, serial measurements of exhaled nitric oxide fraction have the potential to provide a very sensitive strategy for early detection of emerging airway inflammation and subsequent changes in airway hyperresponsiveness to histamine.

KEYWORDS: Adenosine 5′-monophosphate, airway hyperresponsiveness, allergic asthma, exhaled nitric oxide, leukotrienes, prostaglandins

Repeated low-dose allergen inhalation challenge has been introduced as a method to mimic and standardise natural exposure to environmental allergens [1]. In this challenge setting, patients with atopic asthma were subjected to inhalations of fixed doses of allergen, titrated to cause minimal bronchoconstriction and administered once daily on 4–10 consecutive weekdays [1–6]. The procedure generates a distinct increase in airway hyperresponsiveness (AHR) to direct bronchoconstrictors [1–6]. The increase in AHR occurs despite only a few symptoms of asthma being reported by the subjects. The challenge model is, therefore, particularly suitable to investigate early events in the development of AHR, a central feature of the asthmatic phenotype. The relevance of the model is supported by the established effects of inhaled corticosteroids on AHR and sputum eosinophilia induced by the low-dose challenge procedure [7, 8].

However, investigations of mechanisms of airway inflammation in this particular model have been rather limited so far, and findings in peripheral blood are largely negative [5, 7, 9]. In a diluent controlled evaluation of the challenge, the percentage of eosinophils, interleukin-5 and eosinophil cationic protein in induced sputum were shown to increase [2], and early effects on eosinophils and macrophages in bronchoalveolar lavage fluid have also been reported [9, 10]. The aim of the current study was to further characterise the influence of repeated low-dose allergen challenge on the development of airway inflammation. The primary end-point was to establish if there was an association between exhaled nitric oxide fraction ($F_{e,NO}$) and changes in airway responsiveness during repeated low-dose allergen exposure. This would provide evidence on whether or not $F_{e,NO}$ is an appropriate early marker of airway inflammation that may precede symptomatic exacerbations of asthma. Previous data suggest increased $F_{e,NO}$ during low-dose allergen exposure [8], but in that particular study, the increase in $F_{e,NO}$ was not

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Received:
December 14 2005
Accepted after revision:
February 14 2006

SUPPORT STATEMENT
The present study was supported by the Karolinska Institutet, the Centre for Allergy Research and the Stockholm County Council (ALF; all Stockholm, Sweden). Support was also received from the following Swedish foundations: Heart Lung Foundation, Association Against Asthma and Allergy, Consul Bergh's Foundation, Medical Research Council (projects No. 07919, 14X-9071 and 74X-15067), The European Space Agency and the Foundation for Health Care Sciences and Allergy Research (Vårdal foundation). No support was received from the pharmaceutical industry or from Aerocrine AB.

European Respiratory Journal
Print ISSN 0903-1936
Online ISSN 1399-3003

associated with an increase in airway responsiveness to methacholine, nor was diluent control used.

As a secondary surrogate marker of inflammation, urinary levels of leukotriene E_4 (LTE_4) were measured along with urinary levels of the prostaglandin D_2 metabolite, 9α11β-prostaglandin F_2 (PGF_2), which serves as a specific marker of mast cell activation [11]. For example, the allergen-induced early and late asthmatic response in the conventional high-dose allergen challenge is associated with an increase in urinary levels of 9α11β-PGF_2 [12]. While the activation of eosinophils has been shown to occur in the low-dose challenge [2, 7, 8], the activation of mast cells has been less explored. It is becoming increasingly evident that the pathogenetic role of the eosinophil in asthmatic airway inflammation is uncertain [13]. The current authors hypothesised that priming of mast cells takes place during the development of AHR and that measurements of urinary excretion of 9α11β-PGF_2 could test this hypothesis. As another attempt to test whether mast cells were activated, challenges with adenosine 5′-monophosphate (AMP) were included in the protocol. AMP is an indirect bronchoconstrictor which, by acting primarily on mast cells, causes release of bronchoconstricting mediators [14–16]. Responsiveness to AMP has been suggested to be more closely associated with airway inflammation than challenges with direct bronchoconstrictors, such as methacholine [17]. Population studies have shown a relationship between sensitisation to common aeroallergens and AMP responsiveness [18]. The effects of the low-dose allergen challenge procedure on responsiveness to AMP have not been assessed previously.

Thus, with a two-period, crossover design, subjects allergic to pollen or animal dander and with no current asthma symptoms, were exposed to low doses of inhaled allergen and its diluent, respectively, for 7 consecutive weekdays. Bronchial responsiveness to histamine and AMP was assessed before and after the two challenge periods; urinary mediator metabolites were also measured and determinations of $F_{e,NO}$ were performed throughout the respective periods. In addition, a control group of eight healthy individuals was subjected to diluent challenge, using the same study protocol, to establish if baseline $F_{e,NO}$ values and urinary markers differ over time between subjects with mild asthma and healthy individuals.

METHODS

Subjects

Eight nonsmoking subjects with a history of asthma symptoms on exposure to pollen or animal dander were recruited to the study (table 1). They were required to have a positive screening histamine challenge with a provocative dose causing a 20% fall in forced expiratory volume in one second (PD_{20}) ranging 110–2,090 μg and a positive skin-prick test to birch pollen, timothy grass pollen and/or animal dander, but not to house dust mite. Pollen-sensitive subjects were investigated outside the pollen season and subjects sensitive to animal dander did not have pets of their own and were asked to strictly avoid animal contact. Therefore, on entering the study, subjects were asymptomatic, their asthma had been stable and they had had no respiratory tract infection for the last 4 weeks. Their sole medication was occasional use of β_2-agonists. A screening allergen challenge was performed with the allergen considered of most clinical importance in each individual. The subject's current sensitivity was expressed as PD_{20} (table 1). Four out of the eight subjects displayed a dual (early and late) response.

A group of eight nonsmoking, age- and sex-matched healthy control subjects without a history of allergy or asthma were included (seven females and one male; aged 24–43 yrs; mean (range) baseline forced expiratory volume in one second (FEV_1) 106 (93–125)% predicted). All control subjects were skin-prick test negative and displayed a negative screening histamine challenge (PD_{20} >2,090 μg).

The study protocol was approved by the Local Ethics Committee of Karolinska Hospital (Stockholm, Sweden; Dnr: 98:248), and all subjects gave their informed consent to participate. All eight asthmatic subjects and eight control subjects completed the study.

Study design

Four weeks after the screening allergen challenge, the asthmatic subjects entered a two-period, crossover repeated low-dose inhalation challenge with allergen and diluent,

TABLE 1 Characteristics of subjects with asthma

Subject no.	Age yrs	Sex	FEV_1 % pred	PD_{20} histamine μg	Allergen	SPT mm	PD_{20} allergen SQ units	Late reaction	PD_5 allergen SQ units
1	32	F	85	620	Birch	5.5	500	No	100
2	25	F	96	788	Cat	9.5	350	No	14
3	40	F	106	1864	Cat	9.5	1525	Yes	213
4	29	F	81	420	Birch	6.5	500	Yes	21
5	29	F	89	128	Cat	12.0	170	Yes	7
6	41	F	103	197	Timothy	14.5	27	No	7
7	32	F	98	186	Cat	8.5	350	Yes	112
8	26	M	102	1069	Cat	14.5	270	No	42
Mean (range)			95 (81–106)	457 (128–1864)#			295 (27–1525)#		33 (7–213)#

FEV_1: forced expiratory volume in one second; % pred: per cent predicted; PD_{20}: provocative dose causing a 20% fall in FEV_1; standardised quality; SPT: skin-prick test mean diameter; PD_5: provocative dose causing a 5% fall in FEV_1; F: female; M: male. #: geometric mean.

TABLE 2 Schedule for each study period

Visit 1 Thurs.	Visit 2 Fri.	Visit 3 Mon.	Visit 4 Tues.	Visit 5 Wed.	Visit 6 Thurs.	Visit 7 Fri.	Visit 8 Mon.	Visit 9 Tues.	Visit 10 Wed.	Visit 11 Thurs.
$F_{e,NO}$	$F_{e,NO}$	$F_{e,NO}$	$F_{e,NO}$	$F_{e,NO}$	$F_{e,NO}$	$F_{e,NO}$	$F_{e,NO}$	$F_{e,NO}$	$F_{e,NO}$	$F_{e,NO}$
FEV_1	FEV_1	FEV_1	FEV_1	FEV_1	FEV_1	FEV_1	FEV_1	FEV_1	FEV_1	FEV_1
PD_{20} Hi	PD_{20} AMP urine	Ag/Dil #1 urine	Ag/Dil #2	Ag/Dil #3	Ag/Dil #4	Ag/Dil #5	Ag/Dil #6	Ag/Dil #7 urine	PD_{20} Hi	PD_{20} AMP urine

Thurs.: Thursday; Fri.: Friday; Mon.: Monday; Tues.: Tuesday; Wed.: Wednesday; $F_{e,NO}$: exhaled nitric oxide faction; FEV_1: forced expiratory volume in one second; PD_{20}: provocative dose causing a 20% fall in FEV_1; Hi: histamine; AMP: adenosine 5′-monophosphate; Ag/Dil: allergen or diluent inhalation.

respectively. A summary of the study protocol is presented in table 2.

As the current authors original description of the low-dose challenge [1] suggested that the increase in airway responsiveness might take some weeks to resolve, it was decided to perform the diluent challenge single-blindly in the first period followed by the allergen challenge period after a 4 week washout. However, the subjects, the study technician measuring $F_{e,NO}$, and the investigators analysing urinary mediators were unaware of the predefined sequence and the study procedure was identical during the respective period.

First, a histamine challenge was performed followed the next day by an AMP challenge. After the weekend, always starting on a Monday, the repeated low-dose allergen/diluent (Ag/Dil) inhalation period started. On the day after the last Ag/Dil inhalation, a second histamine challenge was carried out and on the following day a second AMP inhalation challenge.

The control subjects were subjected to the same procedure but only received the diluent challenge.

Inhalation challenges

Subjects reported to the clinic at the same time in the morning (07:30–08:00 h) on all challenge days. β_2-Agonists were not allowed for 8 h prior to the challenge. Lung function was measured as FEV_1 on a spirometer (Vitalograph MDI Compact; Förbandsmaterial, Stockholm, Sweden) and the baseline, defined as the best of three recordings, had to be $\geqslant$70% pred. Allergen, histamine and AMP provocation tests were performed by the use of a dosimeter-controlled jet nebuliser (Spira Elektro 2; Respiratory Care Center, Hameenlinna, Finland) as previously described [19]. Challenges were started by the inhalation of diluent. Provided FEV_1 did not change by >10%, the rising dose bronchoprovocation with the respective active substance was started and the post-diluent FEV_1 value was used as baseline. At the screening allergen challenge, half-log increments in the cumulated dose of allergen (range 7–7,100 SQ Aquagen™; ALK, Copenhagen, Denmark) were inhaled every 15 min until a 20% fall in FEV_1 was obtained. Bronchial responsiveness to histamine (histamine diphosphate prepared by the Karolinska Hospital Pharmacy) was assessed with a similar protocol, but with dose increments every third minute. Two concentrations (1.6 $mg \cdot mL^{-1}$ and 16 $mg \cdot mL^{-1}$) and a variable number of breaths were used to create increasing cumulative doses (range 11–2,090μg) of the agonist [19]. The AMP challenges employed a protocol using doubling concentrations every 5 min (1.56–400 $mg \cdot mL^{-1}$; Sigma Chemical Co., St Louis, MO, USA) [20]. The PD_{20} values for allergen, histamine and AMP were calculated by linear interpolation from the log dose–response curves.

Low-dose allergen inhalation challenge

The dose of allergen causing an early fall in FEV_1 of ~5% (PD_5) was determined from the screening allergen challenge in each individual (table 1). The allergen PD_5 was then administered as a single challenge every day for 7 successive weekdays, with a break for the weekend (table 2). The asthmatic patients inhaled the diluent by the same number of breaths as the allergen dose during the respective challenge periods, whereas the control subjects inhaled the diluent by three breaths. Spirometry recordings were taken before and 15 min after inhalation. Patients recorded morning and evening peak expiratory flow rate (PEFR) values during the study (Jaegher electronic peak flow meter, Hoechberg, Germany), and were requested to make further recordings in the event of asthma symptoms. Day- and night-time asthma symptom scores (0=no symptoms; 1=mild symptoms; 2=moderate symptoms; 3=severe symptoms) and short-acting β_2-agonist usage were also recorded on each study day.

Nitric oxide measurements in exhaled air

$F_{e,NO}$ was measured according to the recommendations for online nitric oxide (NO) measurements published by the American Thoracic Society [21], using an Aerocrine prototype NO system (Aerocrine AB, Stockholm, Sweden), including a CLD 77 AM chemiluminescence analyser (Eco Physics AG, Durnten, Switzerland; sensitivity 0.1 ppb NO; rise time 0–90% <0.1 s; sample flow rate 110 $mL \cdot min^{-1}$; lag time from mouthpiece 0.7 s) for online NO measurements, and a pneumotachygraph for monitoring of flow and pressure. Exhalation rate (250 $mL \cdot s^{-1}$) was kept constant by visual feedback during exhalation at 5 cm H_2O through a linear flow resistor (Hans Rudolph Inc., Kansas City, KS, USA). A two-point calibration was performed before each study session using mass-flow controlled dilutions of certified calibration gas (stock concentration 2 ppm NO in N_2; AGA AB, Älvsjö, Sweden).

Urine analyses

On the AMP challenge days, urine was collected upon arrival just before the start of the challenge and 1 h after the last dose of AMP. Likewise, a pre-dose urine sample was obtained on challenge days 1 and 7 during each repeated low-dose period,

whereas the post-dose sample was taken 30 min after the inhalation. The samples were frozen and the concentrations of 9α11β-PGF_2 and LTE_4 were determined according to a validated semi-automated enzyme immunoassay (Cayman Chemical, Ann Arbor, MI, USA) methodology [22, 23].

Statistical analyses

For the primary outcome the sample size of eight subjects was based on the current authors' own measurements allowing for the detection of a 50% increase in $F_{e,NO}$ with 80% power and $\alpha=0.05$. This is in agreement with the publication by KHARITONOV *et al.* [24] on the repeatability and sample size estimates for $F_{e,NO}$ measurements.

Calculations of geometric mean PD_{20}, PD_{10} (dose of allergen causing an early fall in FEV_1 of ~10%) and PD_5 values were performed on log-transformed raw data. The data for pulmonary function, NO values and urinary mediators were found to be normally distributed, and ANOVA and paired t-tests were used to compare different periods and different treatment groups. Data are generally presented as mean±SEM. Differences were considered to be significant when the p-value was <0.05.

RESULTS

Lung function

The repeated low-dose allergen challenge did not affect baseline pulmonary function in the subjects with asthma. Therefore, FEV_1 values obtained each day before low-dose allergen inhalation were no different from values during the diluent period (fig. 1a). Baseline values on the respective histamine and adenosine challenge days were also stable throughout the whole study and showed no significant variability between visits (p=0.16).

None of the subjects had an early asthmatic response following the low-dose allergen challenge. The group mean changes in FEV_1 during each challenge day are displayed in figure 1b with the mean±SEM immediate fall in FEV_1 during the allergen challenge and diluent period being 0.6±1.0% and 0.3±0.7%, respectively. In addition, no subject exhibited a clinically significant late fall in lung function as assessed by PEFR measurements and symptom reporting during the respective challenge period (data not shown).

The group of healthy controls had normal lung function that did not change during the observation period (FEV_1 104±13.6 *versus* 105±13.9% pred on challenge days 1 and 7, respectively, p=0.39).

Symptoms

None of the subjects reported any night-time symptoms of asthma or use of β_2-agonists during either challenge period. During the diluent period no subject reported any symptoms of asthma (symptom score=0) whereas during the low-dose allergen period four subjects reported mild symptoms (symptom score=1) on occasional days (group mean±SEM symptom score: 0.16±0.07).

Airway responsiveness to histamine

Repeated low-dose allergen inhalation produced an increase in the airway responsiveness to histamine in all eight subjects (fig. 2). There was a significant reduction in geometric mean

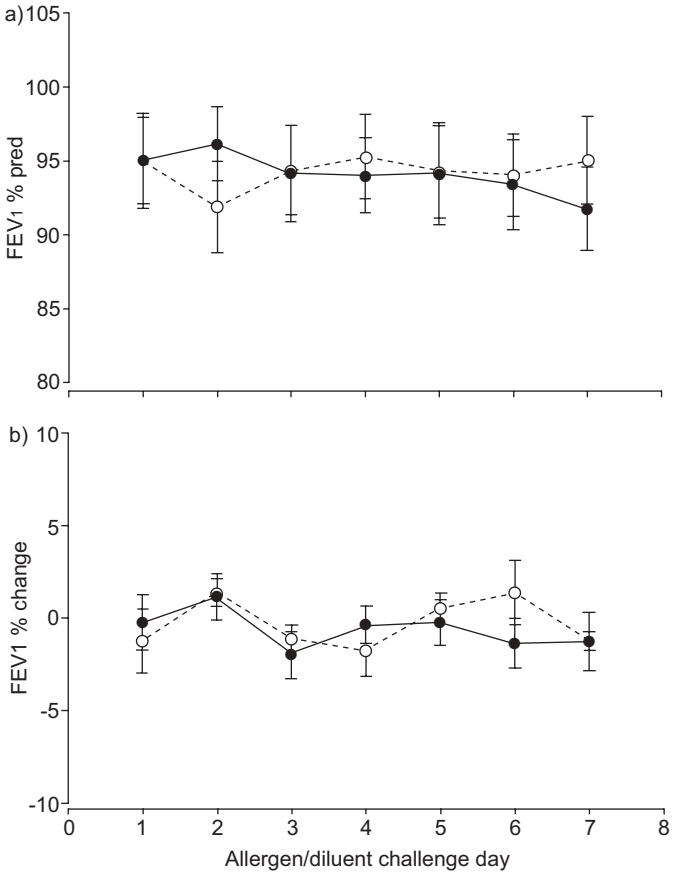

FIGURE 1. Pulmonary function in asthmatic subjects. a) Baseline forced expiratory volume in one second (FEV_1) and b) per cent change in FEV_1 15 min after dosing during the diluent (○) and the repeated low-dose allergen (●) challenge period.

histamine PD_{20} following the allergen period (724 (324–1,622)μg before *versus* 316 (166–603)μg after; p<0.01), corresponding to 2.3 doubling doses. In contrast, histamine PD_{20} was unaffected by repeated doses of the diluent (457 (178–1,175)μg before *versus* 562 (302–1,047)μg after; p=0.48; fig. 2). While there was no variability in pre-challenge histamine responsiveness between the two periods (p>0.05), the shift in histamine PD_{20} was significantly different between the challenge periods (log shift histamine PD_{20} -0.36 *versus* 0.09 for allergen and diluent, respectively; p<0.01).

Exhaled NO

The group $F_{e,NO}$ increased from 8.6±1.4 ppb before the allergen challenge period to 14.7±2.3 ppb 24 h after the last allergen inhalation (p<0.05; fig. 3). In contrast, there was no significant change in $F_{e,NO}$ during the diluent period (9.8±1.7 ppb before *versus* 10.4±1.6 after; p>0.05). However, when assessed for the individuals there was no significant mathematical correlation between the change in NO levels during the allergen period and the corresponding decrease in histamine PD_{20} values (r=0.394; p=0.33).

There were gradually increasing levels of $F_{e,NO}$ in exhaled air during the low-dose allergen period, in contrast to the diluent period where the levels remained stable (fig. 4). The levels of

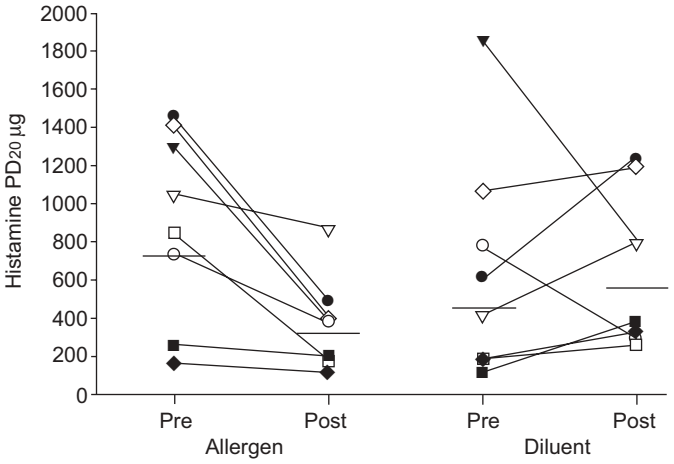

FIGURE 2. Individual and geometric mean histamine provocative dose causing a 20% fall in forced expiratory volume in one second (PD_{20}) values before and after the respective allergen and diluent challenge periods in subjects with asthma. Between changes during diluent *versus* allergen challenge $p<0.05$, as well as before and after the allergen challenge period. Symbols represent individual subjects.

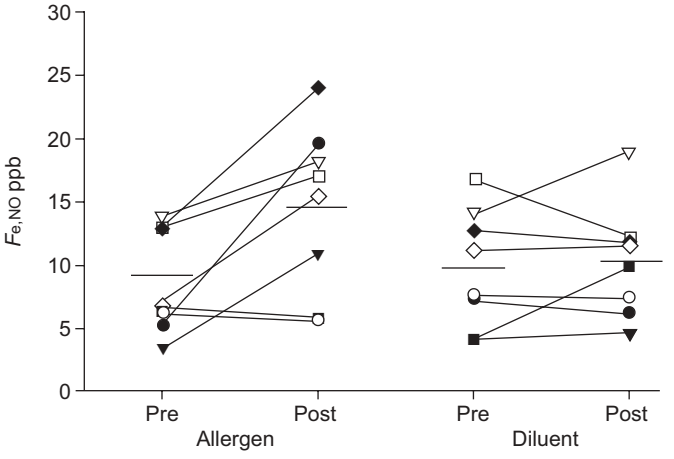

FIGURE 3. Individual and group mean concentrations of exhaled nitric oxide before and after the respective allergen and diluent challenge periods. $F_{e,NO}$: exhaled nitric oxide fraction. Between changes during diluent *versus* allergen challenge $p<0.05$, as well as before and after the allergen challenge period. Symbols represent individual subjects.

$F_{e,NO}$ were also stable in the healthy control group (fig. 4). However, the mean levels of $F_{e,NO}$ during the observation period were significantly lower in the healthy control group in comparison with the asthmatic subjects ($p<0.05$; fig. 4).

Interestingly, the coefficient of intra-individual variability of $F_{e,NO}$ in the asthmatic subjects was 20.7 (6.7–37)% during the allergen challenge period and 12.8 (6.7–31)% during the diluent period, similar to the coefficient of variability for $F_{e,NO}$ in the healthy subjects (14.2 (9.2–25)%).

Airway responsiveness to adenosine

While all eight subjects with asthma by design were hyperresponsive to histamine, only four produced a PD_{20} for AMP of $<1{,}600$ mg·mL^{-1} at inclusion. In these subjects, there

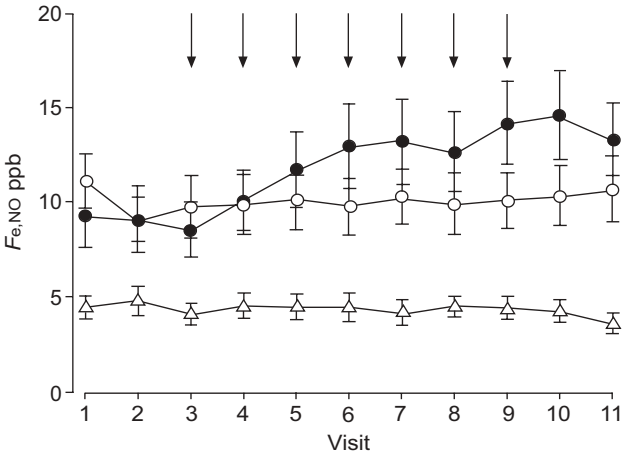

FIGURE 4. Time course of exhaled nitric oxide fraction ($F_{e,NO}$) measurements taken before allergen or diluent inhalation during the respective challenge periods. Arrows denote allergen or diluent challenge days. ●: allergen in asthmatic subjects; ○: diluent in asthmatic subjects; △: diluent in healthy control subjects.

was no significant increase in responsiveness to AMP 48 h after repeated allergen challenge in comparison to the diluent period (log shift PD_{20} AMP being -0.37 and 0.17 after allergen and diluent, respectively; $p=0.14$). It was possible to calculate PD_{10} AMP in seven subjects, but the shift in AMP responsiveness during the allergen period was not significantly different from the diluent period (log shift PD_{10} AMP -0.18 *versus* 0.17 after allergen and diluent, respectively; $p=0.06$).

Urinary mediators

Baseline morning concentrations of urinary 9α11β-PGF_2 and LTE_4 remained unaffected during either challenge period (table 3). However, the asthmatic subjects had significantly higher baseline concentrations of urinary 9α11β-PGF_2 (fig. 5a) and LTE_4 (fig. 5b) compared with the healthy control subjects.

There was no increase in the urinary excretion of either mediator after the allergen challenges on day 1 and 7 (table 3). There was, however, an increase in urinary excretion of 9α11β-PGF_2 after adenosine challenge at the end of the repeated allergen exposure period, but not at the end of the diluent period (table 3). Urinary levels of LTE_4 were unchanged after adenosine challenge during both periods (table 3).

DISCUSSION

The present study documented increased airway responsiveness to histamine following seven repeated low-dose allergen exposures during a 9-day period in subjects with mild asthma. At the same time, the subjects had no symptoms of asthma and did not need to use rescue bronchodilator medications. Furthermore, the increased airway responsiveness was obtained despite the subjects having no change in baseline airway calibre. The study, therefore, adds to the indications that the low-dose allergen challenge method is well suited to studying both the early events in allergic airway inflammation and the mechanisms involved in AHR. In contrast to most previous studies using different protocols for low-dose allergen challenge, the current authors included a diluent period as it was felt that subjects with asthma, according to the

TABLE 3 Excretion of mediators in urine[#]

	Diluent period		Allergen period	
	9α11β-PGF_2	LTE_4	9α11β-PGF_2	LTE_4
Before AMP visit 2	77±8	50±8	101±28	58±9
After AMP visit 2	83±6	47±5	110±30	51±5
Before Ag/Dil visit 3	76±12	58±18	74±8	46±6
After Ag/Dil visit 3	82±13	61±14	94±21	49±3
Before Ag/Dil visit 9	83±9	44±5	84±12	49±4
After Ag/Dil visit 9	79±7	53±8	85±13	53±4
Before AMP visit 11	101±10	71±18	77±10	46±4
After AMP visit 11	119±30	57±3	101±13*	46±4

Data are presented as mean±SEM ng·mmol^{-1} creatinine. 9α11β-PGF_2: 9α11β-prostaglandin F_2; LTE_4: leukotriene E_4; AMP: adenosine 5′-monophosphate; Ag/Dil: antigen or diluent inhalation. #: asthmatic subjects, n=8. *: $p<0.05$ *versus* value before challenge, p-value for all other pre- and post-comparisons were >0.05.

natural course of the disease, may display increased spontaneous variability in airway responsiveness and inflammation. Furthermore, a group of healthy individuals inhaling diluent for a similar challenge period were also included. Another measure to enhance the resolution of the model was to minimise the accidental environmental exposure to the study allergens. Therefore, the present authors studied subjects out of their season and specifically excluded house dust mite allergic subjects as elimination of that particular natural exposure is very difficult and presumably introduces a confounding background trigger of inflammation in many studies.

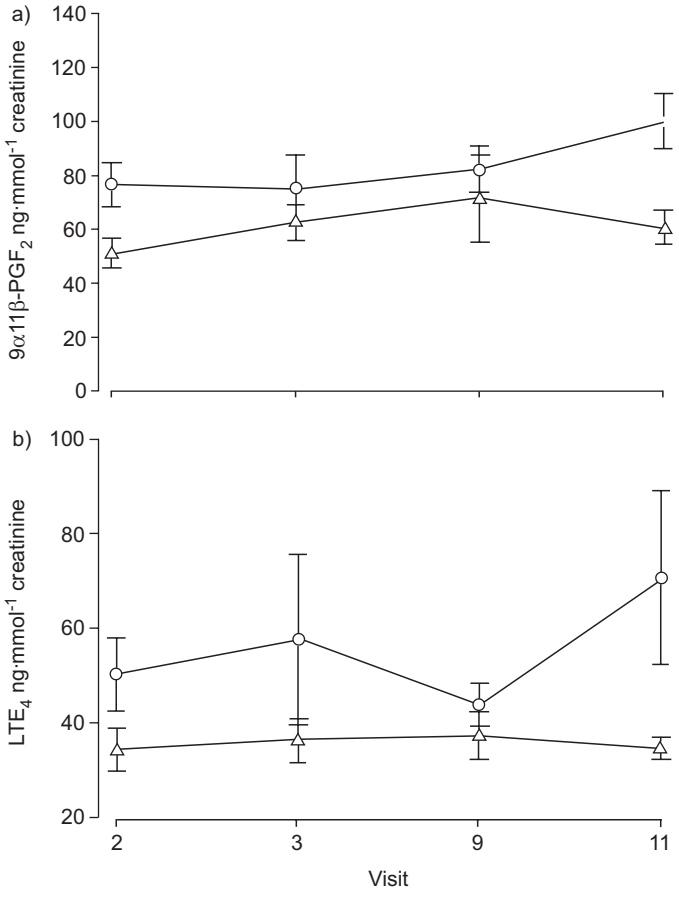

FIGURE 5. Baseline urinary concentrations of a) 9α11β-prostaglandin F_2 (9α11β-PGF_2) and b) leukotriene E_4 (LTE_4) on visits 2, 3, 9 and 11 in subjects with asthma (○) and in healthy controls (△) during the diluent period. Between mean values of the respective mediator level $p<0.05$.

The main finding in the study was that of a progressive increase in NO levels in exhaled air during the repeated low-dose allergen exposures. Thus, the mean value of $F_{e,NO}$ was almost doubled after the allergen challenges, whereas the levels of $F_{e,NO}$ were unchanged and stable after repeated challenge with the diluent in the same subjects. The current finding confirms and extends the reports from one group of investigators of increased exhaled NO levels after repeated low-dose allergen challenge [8, 25]. However, the increase in exhaled NO was only associated with increased AHR to methacholine in one of the studies [25]. As the subjects in the current study had no symptoms of asthma nor changes in lung function, the finding supports the hypothesis that increased $F_{e,NO}$ may be an early and very sensitive sign of increased airway inflammation. It has been documented that measurements of $F_{e,NO}$ at 4–8-week intervals may improve the dosing of inhaled steroids required to achieve asthma control [26]. An early and progressive rise of $F_{e,NO}$ was found within a few days after the start of the allergen challenge period. This suggests that monitoring $F_{e,NO}$ on a daily basis might represent a strategy for early detection of exacerbations, providing that there is the opportunity for treatment before the exacerbation has reached the ''point of no return'' where no treatments so far have been able to abrogate an exacerbation [27].

The present study also showed that even subjects with mild untreated asthma had higher baseline levels of $F_{e,NO}$ than a group of healthy controls. During the diluent period, the asthmatic subjects nevertheless had stable levels of $F_{e,NO}$ and the day-to-day variability was similar to that of the healthy controls. In contrast, during the allergen period, the variability in $F_{e,NO}$ levels increased, suggesting that another early sign of increased airway inflammation may be increased day-to-day variability in $F_{e,NO}$.

The secondary end-point in the study was to evaluate the usefulness of monitoring urinary levels of LTE_4 and the mast cell marker 9α11β-PGF_2. It was found that when assessed on four occasions during a 10-day period, the asthmatic subjects had elevated baseline levels of both mediators in the urine compared with the healthy controls. Although the difference was small, it was significant and indicates that by performing repeated sampling one may detect differences even in relatively small groups of subjects. Most studies that have failed to find significant differences in urinary LTE_4 between healthy subjects and subjects with mild asthma have compared cross-sectional data comprised of single measurements that clearly have insufficient power to detect small differences [28].

However, in the subjects with asthma there was no progressive change in baseline urinary excretion of either mediator during the allergen challenge period. The only significant difference that was found at the group level was an increase in 9α11β-PGF_2 excretion after the AMP challenge 48 h after the allergen

period. Urinary 9α11β-PGF_2 is established as the most sensitive method for detection of mast cell activation [11]. Therefore, the finding of increased 9α11β-PGF_2 supports priming of the mast cells during the allergen period or increased infiltration of mast cells, in particular as the dose of AMP was similar before and after the period. The current observation suggesting increased mast cell activation may relate to the report of increased numbers of methachromatic cells in sputum in another low-dose allergen challenge study [2], incidentally the only other study that has used a diluent control arm. Admittedly, the effect the present authors observed of AMP on prostaglandin D_2 (PGD_2) release was small, and further studies are required to establish this mechanism. As the AMP provocations were carried out 48 h after the last allergen inhalation, it might also be that the peak of increased mast cell activation was missed. In addition, given that the predetermined PD_5 dose resulted in only small falls in FEV_1, it is conceivable that larger repeated allergen doses might have produced significant changes in urinary markers or in airway responsiveness to AMP.

One reason for the modest increase in the mast cell product PGD_2 in the current study might relate to the relative insensitivity to AMP that was obtained with the protocol used. Thus, only four out of eight subjects produced a PD_{20} for AMP before the study period. As AMP challenges have been proposed to be very specific for asthma, it is a little surprising that only half of the subjects in a group with mild asthma displayed significant responsiveness to AMP. However, the current protocol for AMP challenges may not have been optimal.

Obviously, the present data support the conclusion that increased $Fe_{,NO}$ is a more sensitive and early marker of airway inflammation than urinary LTE_4. In fact there may be a mechanistic explanation of the unchanged levels of urinary LTE_4 in the current study. Thus, it was recently observed in a model of the peripheral lung that NO specifically inhibits allergen-induced release of leukotrienes [29]. It might be that the increased levels of NO during early airway inflammation represent an important protective mechanism intended to limit the development of the inflammation.

In conclusion, while the debate on whether nitric oxide is predominantly pro- or anti-inflammatory continues, the present study undoubtedly supports the fact that frequent serial measurements of exhaled nitric oxide fraction have the potential to provide a very sensitive strategy for early detection of emerging airway inflammation. Moreover, the low-dose allergen challenge model [30] is here to stay as it provides opportunities for safe and controlled investigations of mechanisms in airway hyperresponsiveness and airway inflammation in the absence of baseline bronchoconstriction and ongoing asthmatic symptoms.

ACKNOWLEDGEMENTS

The authors would like to thank A. Guhl, I. Delin, M. Andersson, B. Rasberg, H. Blomqvist and C. Larsson for their technical assistance.

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